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Characterization of *cis*-regulatory Elements of the c-*myc* Promoter Responding to Human GM-CSF or Mouse Interleukin 3 in Mouse proB Cell Line BA/F3 Cells Expressing the Human GM-CSF Receptor

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Interleukin 3 (IL-3) or granulocyte macrophage colony-stimulating factor (GM-CSF) activates c-*fos*, c-*jun*, and c-*myc* genes and proliferation in both hematopoietic and nonhematopoietic cells. Using a series of deletion mutants of the β subunit of human GM-CSF receptor (hGMR) and inhibitors of tyrosine kinase, two distinct signaling pathways, one for activation of c-*fos* and c-*jun* genes, and the other for cell proliferation and activation of c-*myc* gene have been elucidated. In contrast to wealth of information on the pathway leading to activation of c-*fos*/c-*jun* genes, knowledge of the latter is scanty. To clarify the mechanisms of activation of c-*myc* gene by cytokines, we established a transient transfection assay in mouse proB cell line BA/F3 cells expressing hGMR. Analyses of hGMR β subunit mutants revealed two cytoplasmic regions involved in activation of the c-*myc* promoter, one is essential and the other is dispensable but enhances the activity. These regions are located at the membrane proximal and the distal regions covering amino acid positions 455–544 and 544–589, respectively. Characterization of *cis*-acting regulatory elements of the c-*myc* gene showed that the region containing the P2 promoter initiation site is sufficient to mediate the response to mIL-3 or hGM-CSF. Electrophoretic mobility shift assay using an oligonucleotide corresponding to the distal putative E2F binding site revealed that p107/E2F complex, the negative regulator of E2F, decreased, and free E2F increased after mIL-3 stimulation. These results support the thesis that mIL-3 or hGM-CSF regulates the c-*myc* promoter by altering composition of the E2F complexes at E2F binding site.

INTRODUCTION

The c-*myc* gene was originally identified as a cellular homologue of the v-*myc* gene, an oncogene responsible for chicken leukemia (Sheiness and Bishop, 1979). It is an essential gene because disruption of c-*myc* gene resulted in embryonic lethality (Davis *et al.*, 1993). c-*myc* protein is a transcription factor containing basic helix-loop-helix and leucine zipper motifs (Blackwell *et al.*, 1990; Murre *et al.*, 1989). It forms a dimer with another protein, Max (Blackwood *et al.*, 1991), and this complex binds to a specific DNA sequence and activates transcription. The sequence recognized by c-*myc* protein proved to be a short stretch that is part of the E-box consensus (Blackwell *et al.*, 1990), but knowledge of the target gene(s) of c-*myc* protein is limited. Much attention has been directed to understand the role of c-*myc* protein in regulating proliferation, differentiation, or cell death (Spencer and Groudine, 1991). Deregulation of c-*myc* level results in carcinogenesis or transformation (Hunter, 1991). Modulation of cyclin E expression by c-*myc* protein was reported

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(Hanson *et al.*, 1994) and the role of c-*myc* protein in cell proliferation has been implicated yet the exact function remains to be clarified.

Expression of c-*myc* gene correlates with the cell cycle, i.e., after mitogen stimulation, the c-*myc* mRNA level dramatically increased (Cole, 1986) and, in quiescent cells or during differentiation, it is down regulated (Kelly *et al.*, 1983). Level of c-*myc* mRNA is tightly regulated at multiple states, including initiation and elongation of transcription and mRNA stability (Spencer and Groudine, 1991; Marcu *et al.*, 1992). The human c-*myc* promoter contains two putative E2F-binding sites and disruption of these sites abolishes expression of the c-*myc* gene (Hiebert *et al.*, 1989).

In hematopoietic cells, c-*myc* protein has been assumed to play a role in proliferation, differentiation, and apoptosis (Bentley and Groudine, 1986; Spotts and Hann, 1990). The c-*myc* gene is one of early response genes activated by cytokines such as interleukin 3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Arai *et al.*, 1990; Watanabe *et al.*, 1993a). These cytokines also stimulate other early response genes such as c-*fos*, c-*jun*, and egr and promote proliferation of various types of hematopoietic cells (Miyajima *et al.*, 1992). We previously reported that EGF failed to stimulate proliferation of BA/F3 cells expressing EGFR (Wang *et al.*, 1989). Evidence indicated that hematopoietic cells expressing EGFR proliferate in response to EGF, when a sufficient level of c-*myc* mRNA is present (Shibuya *et al.*, 1992). These results suggest a tight correlation between c-*myc* gene activation and cell proliferation.

Receptors of IL-3 (IL-3R) and GM-CSF (hGMR) are members of a cytokine receptor superfamily (Watanabe *et al.*, 1991; Miyajima *et al.*, 1992) and high affinity and functional human IL-3R/GMR/IL-5R consists of the α subunit specific to each cytokine and a common β subunit (βc subunit) shared by all (Hayashida *et al.*, 1990; Itoh *et al.*, 1990; Kitamura *et al.*, 1991; Hara and Miyajima, 1992). The existence of two distinct signaling pathways, one for activation of c-*fos* and c-*jun* genes and the other for cell proliferation and activation of c-*myc* gene has been postulated, on the basis of findings using a series of deletion mutants of the β subunit in the cytoplasmic domain and tyrosine kinase inhibitors (Sakamaki *et al.*, 1992; Watanabe *et al.*, 1993b). Activation of the c-*myc* gene and cell proliferation required the membrane proximal region of the hGMR β subunit, and tyrosine kinase inhibitors completely suppressed these activities. In contrast, activation of c-*fos*/c-*jun* genes required the cytoplasmic region of the β subunit and was resistant to these inhibitors. Therefore, the hGMR system provided an opportunity to dissect intracellular signals leading to activation of early response genes and to cell proliferation in hematopoietic cells.

IL-3 or GM-CSF has been shown to activate or phosphorylate several signaling molecules such as Ras (Duronio *et al.*, 1989; Satoh *et al.*, 1991), Raf, MAP kinases (Okuda *et al.*, 1992; Welham *et al.*, 1992), shc (Cutler *et al.*, 1993), and PI3′-kinase (Gold *et al.*, 1994; Welham *et al.*, 1994). It is likely that the MAP kinase cascade that has been shown to activate c-*fos* gene in fibroblasts, is also involved in c-*fos* gene activation downstream of GMR. In contrast, signals and pathways downstream of IL-3R or GMR leading to the activation of c-*myc* gene and cell proliferation are largely unknown. Tyrosine kinase inhibitors such as herbimycin or genistein inhibit c-*myc* mRNA induction of BA/F3 cells expressing hGMR after stimulation by mIL-3 or hGM-CSF (Watanabe *et al.*, 1993b). These results suggested the involvement of tyrosine kinases in c-*myc* gene activation, but the nature of the tyrosine kinase remains to be clarified. It is also not clear how activation of the c-*myc* gene is linked to cell proliferation. One of the difficulties in characterizing these pathways is the lack of sensitive and convenient assays. A transient transfection assay of c-*myc* promoter whose expression is controlled by cytokine is not available. The regulation of c-*myc* gene expression has been studied mostly by quantitating the amount of endogenous c-*myc* mRNA by Northern blot analysis. Likewise, cell proliferation has been monitored by incorporation of radioactive thymidine into chromosomal DNA.

To aid the analysis of pathways leading to cell proliferation, we recently established a transient transfection assay in BA/F3 cells expressing hGMR where the polyoma replicon replicates in a manner dependent on mIL-3 or hGM-CSF (Watanabe *et al.*, 1995). To facilitate analysis of the c-*myc* promoter, we describe here a transient transfection assay of the c-*myc* promoter, the activation of which is regulated by mIL-3 or hGM-CSF. Using this system, we characterized the region of the hGMR β subunit involved in activation of the c-*myc* promoter as well as the *cis*-regulatory element of the c-*myc* promoter responding to hGM-CSF signals.

MATERIALS AND METHODS

Chemicals, Media, and Cytokines

Fetal calf serum was obtained from Biocell Laboratories (Carson, CA). RPMI 1640 was obtained from Nikken BioMedial Laboratories. Recombinant hGM-CSF and recombinant mIL-4 produced in *Escherichia coli* were provided by Dr. R. Kastelein, DNAX Research Institute. mIL-3 produced by the silkworm *Bombyx mori* was purified as described elsewhere (Miyajima *et al.*, 1987). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{3}H]chloramphenicol were obtained from Amersham Japan. Geni- stein was obtained from Wako Pure Chemical Industries. Herbimycin, G418, and Opti-MEM were purchased from Life Technologies, Gaithersburg, MD. Anti-p107 (SD9) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Culture Methods

An mIL-3–dependent proB cell line BA/F3 was maintained in RPMI 1640 medium containing 10% fetal calf serum, 1 ng/ml mIL-3, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete media). Transfected BA/F3 cells expressing hGMR α and β subunits (BA/FGMR) were grown in the same type of media but supplemented with 500 μg/ml G418.

Transfection and Chloramphenicol Acetyltransferase (CAT)/Luciferase Assay

After depletion of mIL-3 for 5 h, BA/F3 cells were co-transfected with 20 μg of pmycCAT reporter plasmid and 2 μg each of hGMR α and β subunit plasmids (Sakamaki *et al.*, 1992, Watanabe *et al.*, 1993b) by electroporation, as described elsewhere (Ishida *et al.*, 1994). Briefly, cells suspended in 0.2 ml of Opti MEM (3×10^7 cells) were transferred to a cuvette and mixed with DNA. Cells were electroshocked using 960 μ Farads at 200 V using a Gene Pulser electroporation apparatus (Bio-Rad, Richmond, CA). After 30 min of incubation at room temperature, cells were divided into three portions and transferred to three separate 10-cm dishes and re-stimulated with 5 ng/ml of either mIL-3 or hGM-CSF. After 16 h of incubation, cells were harvested and lysed in 300 μl of 0.25 M Tris, pH 7.4, by sonication and were heat treated at 65°C for 10 min. Each sample containing approximately 50 μg of total protein was subjected to CAT activity assay. CAT activities were analyzed using thin layer chromatography (TLC) to separate the modified from the unmodified substrate as described (Gorman *et al.*, 1982) or by diffusion analysis (Maniatis *et al.*, 1982). TLC was visualized either by autoradiogram or by image analyzer (Fuji BAS 2000). The percent of conversion of modified chloramphenicol was calculated using an image analyzer with optimal calibration range. For c-*fos*–luciferase assay, 2 μg of c-*fos*–luciferase plasmids that contain 0.4-kb c-*fos* promoter upstream of the initiation site and luciferase coding region (Watanabe *et al.*, 1993a), was transfected to BA/FGMR cells and the cells were processed using the same procedure as for the pmycCAT assay. Each sample containing approximately 20 μg of total protein was subjected to luminescence assay. Substrate was automatically injected into the sample in the luminometer (model LE9501; Berthold Lumat) and luminescence of 30 s was counted and expressed as arbitrary units.

Preparation of Nuclear Extract

BA/FGMR cells (5×10^6) were depleted of mIL-3 for 5 h and then stimulated with hGM-CSF or mIL-3 (5 ng/ml). After incubation for the indicated time, these cells were collected and nuclear proteins were extracted as described elsewhere (Watanabe *et al.*, 1993b, 1994).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was made up with nuclear extracts prepared from BA/F3 cells, according to the method described above. myc I (5′CGGAGGGAGGGATCGCGCTG5′), myc II (5′GATCTGCTTGGCGGGAAAAA3′), and E2–1 (5′GATCTGAGAAAGGGCGCGAAACTAGTCCTTA3′) oligonucleotides were chemically synthesized and purified by Sephadex G-50 Quick Spin columns (Boehringer, Mannheim, Germany) after being labeled with [α-^{32}P] dGTP. DNA fragments from plasmids were prepared as follows and purified as described (Ishida *et al.*, 1994). The *Xho*I–*Alu*I sites (X–Al) of the pmycXCAT fragment was isolated and the fragment was further excised with digestion by *Sau*3AI and yielded two fragments, one from the *Xho*I site to the *Sau*3AI site (X–3) and the other from the *Sau*3AI site to the *Alu*I site (3–Al). EMSA was done as described elsewhere (Ishida *et al.*, 1994) using the nuclear extract containing 5 μg protein and labeled oligonucleotides or purified fragment DNA as probes. The gel was transferred to Whatman 3 MM paper, dried, and analyzed by autoradiogram or FUJI Image Analyzer (model BAS-2000).

RESULTS

mIL-3 or hGM-CSF but Not mIL-4 Activated the c-myc Promoter in BA/F3 Cells Expressing hGMR

mIL-3 induces c-*myc* mRNA in various types of hematopoietic cells. We have previously shown that hGM-CSF induces c-*myc* mRNA together with other early response mRNAs such as c-*fos* and c-*jun* through reconstituted hGMR in BA/F3 cells as well as in fibroblasts (Watanabe *et al.*, 1993a). However the nature of signals and the mechanism regulating c-*myc* gene by mIL-3 or hGM-CSF were not well characterized due to the lack of an appropriate assay. To elucidate the mechanism regulating the c-*myc* gene in response to mIL-3 or hGM-CSF stimulation, we tried to design a transient transfection assay using the c-*myc* promoter linked to the CAT reporter gene in the mouse proB cell line BA/F3. BA/FGMR cells express endogenous mIL-3R and mIL-4R, and hGMR whose expression is directed by transfected hGMR cDNA (Hayashida *et al.*, 1990; Watanabe *et al.*, 1993a). Both mIL-3 and hGM-CSF support proliferation of BA/FGMR cells whereas mIL-4 sustains only transient survival up to 24 h but not proliferation of BA/FGMR cells. pmycCAT, which contains the *Hin*dIII site to the *Pvu*II site of the c-*myc* promoter (Figure 1B), was transfected and stimulated either with mIL-4 (10 ng/ml), mIL-3, or hGM-CSF (5 ng/ml). Transfection efficiency of each sample derived from the same transfectant should be the same. After 16 h of incubation, the cells were harvested and CAT activity of the nuclear extracts was determined by TLC, as described in MATERIALS AND METHODS. As shown in Figure 1A, both mIL-3 and hGM-CSF increased CAT activity four- to fivefold over that of the unstimulated control. mIL-4 did not increase c-*myc* CAT activity.

mIL-3 or hGM-CSF Activates the c-myc Promoter through the P2 Region in BA/FGMR Cells

We next characterized *cis*-regulatory elements of the c-*myc* promoter required to mediate the response to mIL-3 or hGM-CSF. Various deletion mutants of the c-*myc* promoter reporter plasmids (Ishida *et al.*, 1994) used in these experiments are schematically illustrated in Figure 1B. CAT activities (% conversion/protein) of the extracts prepared from unstimulated (open bar) or mIL-3–induced (5 ng/ml, hatched bar) BA/FGMR cells transfected with various mutants are shown in the right column. A series of deletions from the 5′ side up to the *Sma*I site (nucleotide position 2225) located close to the P1 starting site (referred to here as the P1 promoter) had no significant effect on the potential to respond to mIL-3 and the levels of induction of these deletion constructs were not appreciably different. Essentially the same results were obtained with hGM-

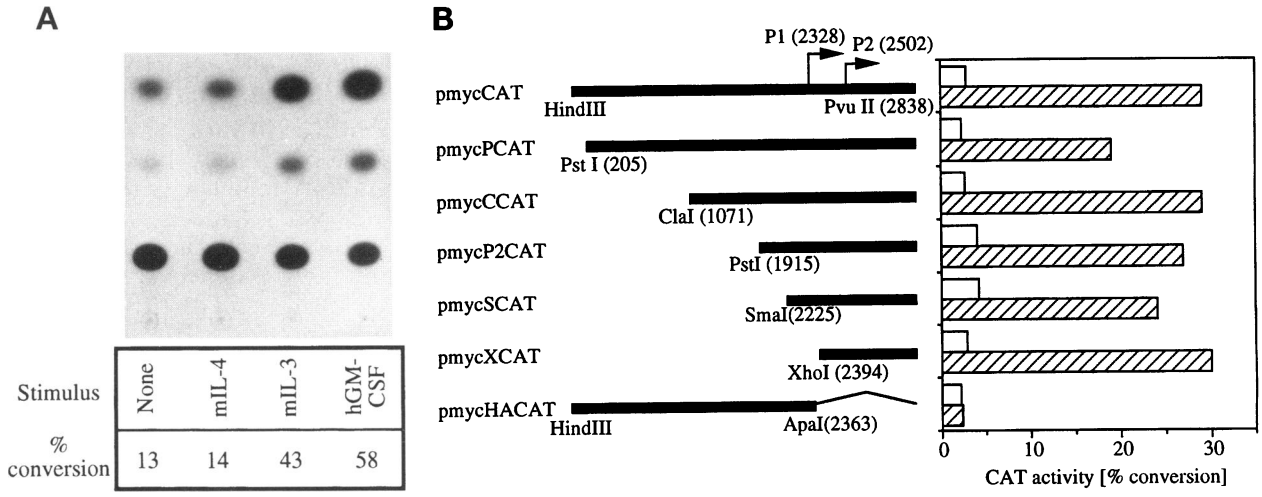


Figure 1. Transient analysis of mIL-3/hGM-CSF induced c-*myc* promoter activity in BA/FGMR cells. pmycCAT (A) or various c-*myc* promoter deletion-CAT plasmids (B) were introduced into BA/FGMR cells by transfection and the cells were depleted of mIL-3 for 5 h, and restimulated with either mIL-4, mIL-3, or hGM-CSF. After 16 h of incubation, cells were harvested and CAT activity that was induced by either mIL-4, mIL-3, or hGM-CSF was analyzed as described in MATERIALS AND METHODS. Panel A shows the TLC pattern and % conversion of acetylated chloramphenicol. Panel B represents schematic illustrations of constructs and CAT activities. Various deletion mutants of the c-*myc* promoter were inserted upstream of CAT. The transcription initiation site of the P1 and P2 promoters are indicated by bent arrows. CAT activities (% of acetylation of chloramphenicol) of each construct induced by mIL-3 (hatched bar) or unstimulated (open bar) in BA/FGMR cells are represented

CSF used as a stimulant. These results suggest that the region 5′ to the side of the P1 promoter is not essential for the activity. It is well documented that transcription of the c-*myc* gene can be initiated via two distinct sites, using either the P1 or P2 promoter (Marcu *et al.*, 1992). There is a report showing that the endogenous c-*myc* starting site is mainly from the P2 promoter (Marcu *et al.*, 1992). To evaluate the contribution of the P1 and P2 promoters in this transient transfection assay, we compared reporter plasmids containing either the P1 and P2 promoters or only the P2 promoter. As shown in Figure 1B, pmycXCAT that lacks the P1 promoter responded to mIL-3 nearly to the same extent as did the plasmid containing both the P1 and P2 promoters. In contrast, the internal deletion construct pmycHACAT, which lacks the P2 promoter region, lost the potential to respond to mIL-3 or to hGM-CSF even though it contains the longest 5′ flanking region among the c-*myc* promoters used. Essentially the same results were obtained by hGM-CSF stimulation. These results, which are consistent with those for the endogenous c-*myc* gene, indicate that mIL-3 or hGM-CSF mainly activates the P2 promoter in the transfection assay.

Effects of mIL-3 or hGM-CSF on Protein-DNA Complexes Formed at the P2 Promoter Region

We next characterized various regions of the c-*myc* promoter in the pmycXCAT plasmid, which has minimal P2 promoter region responding to mIL-3. Nuclear extracts were prepared from unstimulated or mIL-3–stimulated BA/F3 cells, and formation of protein-DNA complexes was analyzed by EMSA, using DNA fragments of pmycXCAT (Figure 2A). As shown in Figure 2B (left panel), three bands were observed when the X-Al probe was used with nuclear extract prepared from unstimulated cells, and the intensity of bands A and C decreased after mIL-3 stimulation. In contrast, the intensity of band B increased slowly and reached a plateau 3 h after mIL-3 stimulation. Likewise, when X-3 was used as a probe, three bands were observed with nuclear extract prepared from unstimulated cells, and the intensity of bands D and E decreased after mIL-3 stimulation. In contrast, the intensity of band F increased after mIL-3 stimulation. When 3-Al fragment was used as a probe, one band was observed, the intensity of which did not change by the addition of mIL-3 (Figure 2B, right panel). Essentially the same results were obtained with hGM-CSF stimulation (data not shown).

Role of E2F Binding Site for Complex Formation at the P2 Promoter Region

Two putative E2F binding sites, mycI and mycII, are present within the region encompassing the *Xho*I and *Alu*I sites of the c-*myc* promoter; only mycII was found to bind purified E2F protein (Hiebert *et al.*, 1989). To examine the role of E2F binding site in the DNA-protein complex formed at the P2 promoter region,

competition assay was done with an unlabeled oligonucleotide carrying the E2F site of the adenovirus E2 promoter (E2–1), or mycI or mycII of the c-*myc* promoter (Figure 2A). E2F and mycII oligonucleotides added in excess (100-fold molar excess) abolished all detectable bands but the mycI oligonucleotide did not affect protein-DNA complexes formed with X-Al or X-3 probes (Figure 2C). Neither E2–1 nor mycII oligonucleotides affected the patterns of bands formed with the 3-Al probe (data not shown). EMSA using labeled mycI oligonucleotide and BA/F3 nuclear extract yielded no detectable band at any time point after mIL-3 stimulation (data not shown). These results indicate that various complexes formed with X-Al and X-3 probes require the mycII site and are likely to involve E2F-related proteins.

Characterization of Protein Complexes Formed at the E2F Site

E2F protein forms a hetero-oligomer with several proteins such as Rb (Nevins, 1992), p107 (Cao *et al.*, 1992), DP-1 (Helin *et al.*, 1993), or cyclins (Mudryj *et al.*, 1991). The E2F/DP-1 heterodimer is capable of activating transcription through the E2F site (Bandara *et al.*, 1993; Helin *et al.*, 1993; Krek *et al.*, 1993). Various E2F hetero-oligomers may differently affect the potential of E2F protein in transcriptional activation (Weintraub *et al.*, 1992). For example, the hetero-oligomer of E2F and Rb protein resulted in loss of activity. To further characterize the protein complexes formed at the E2F site of the c-*myc* promoter, EMSA was done using DOC, which is known to dissociate the E2F hetero-oligomer complex without affecting the free E2F (Baeuerle and Baltimore, 1988; Bagchi *et al.*, 1990). Treatment of the nuclear extract with DOC abolished bands A and C with X-Al as a probe, and bands D and E with X-3 as a probe (Figure 3). The remaining bands B and F correspond to bands increased after mIL-3 stimulation. Taken together, these results mean that the free E2F increased and the E2F hetero-oligomer decreased after mIL-3 stimulation. We next examined the effects of various antibodies on the formation of protein-

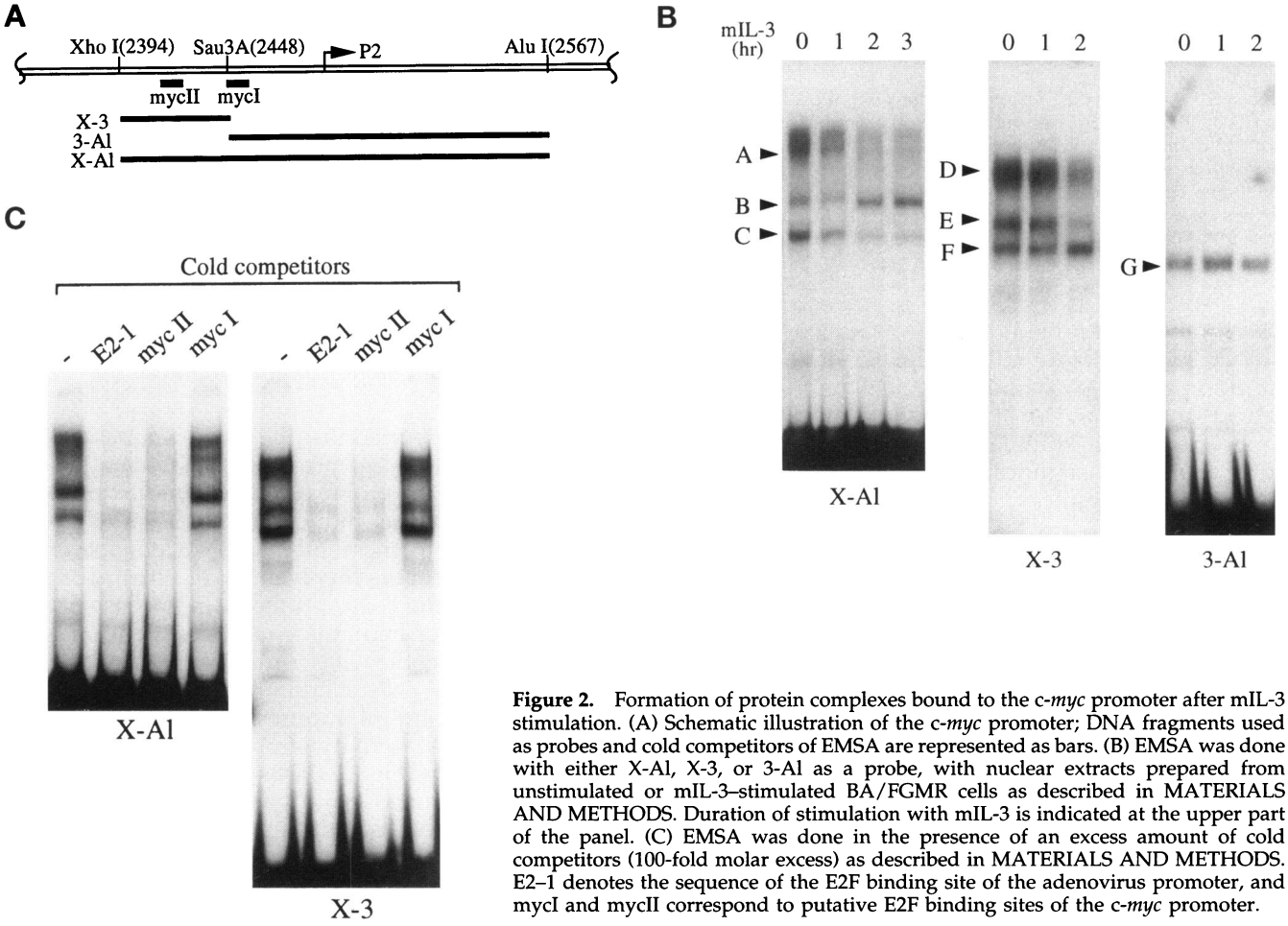


Figure 2. Formation of protein complexes bound to the c-*myc* promoter after mIL-3 stimulation. (A) Schematic illustration of the c-*myc* promoter; DNA fragments used as probes and cold competitors of EMSA are represented as bars. (B) EMSA was done with either X-Al, X-3, or 3-Al as a probe, with nuclear extracts prepared from unstimulated or mIL-3–stimulated BA/FGMR cells as described in MATERIALS AND METHODS. Duration of stimulation with mIL-3 is indicated at the upper part of the panel. (C) EMSA was done in the presence of an excess amount of cold competitors (100-fold molar excess) as described in MATERIALS AND METHODS. E2–1 denotes the sequence of the E2F binding site of the adenovirus promoter, and mycI and mycII correspond to putative E2F binding sites of the c-*myc* promoter.

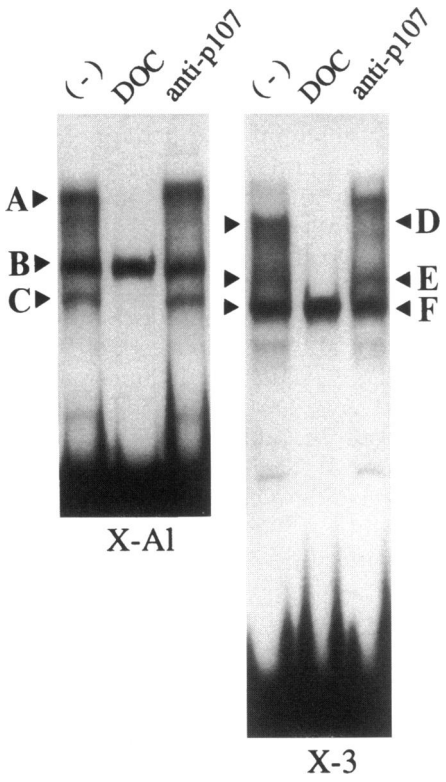


Figure 3. Analysis of E2F complexes detected in BA/F3 cell stimulated by mIL-3. EMSA was done in the presence of DOC (Baeuerle and Baltimore, 1988) or antibody. Anti-p107 antibody (1 μl/lane) was added and the preparation was incubated at room temperature for 20 min before adding the labeled probes, or EMSA was done in the presence of DOC (final 0.4%) and Nonidet P-40 was added at a final concentration of 1% at the end of the reaction.

DNA complex. Antibody (1 μl) was added to nuclear extract 20 min before addition of the labeled probe. Addition of anti-p107 antibody (SD9) to an untreated extract caused band A of X-Al and band D of X-3 to super-shift. With stimulated extract, the same antibody caused no significant change in the pattern of DNA-protein complex.

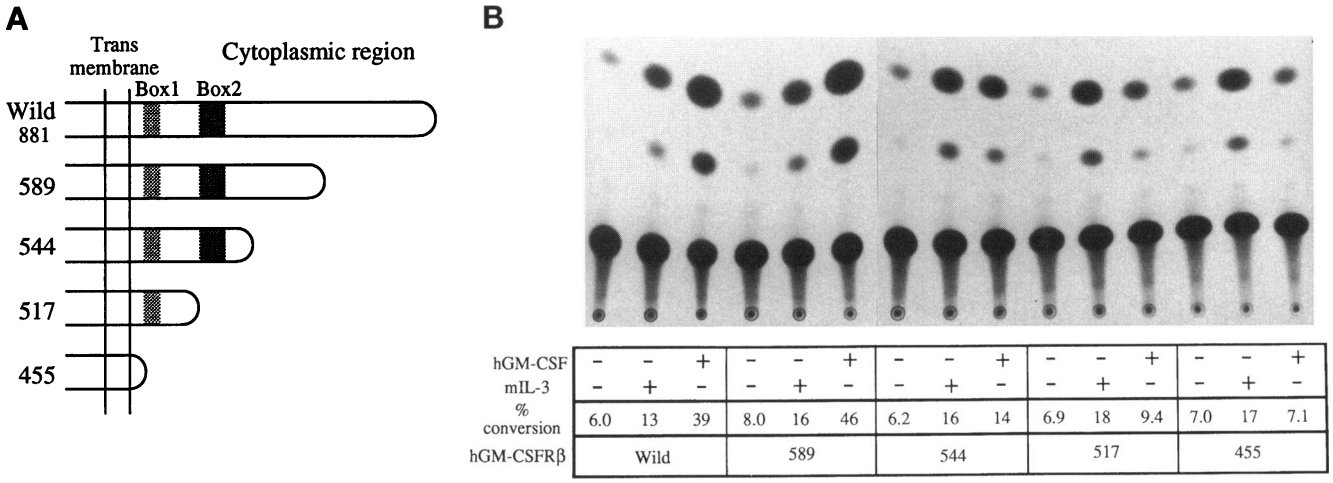
Membrane Proximal Region of hGMR β Subunit Is Required to Activate the c-myc Promoter

We next determined the cytoplasmic region of the β subunit required for activation of the c-*myc* promoter, in response to hGM-CSF. BA/F3 cells were cotransfected transiently with cDNA encoding wild-type hGMR α and various β subunit deletion mutants and the pmycCAT reporter plasmid. A schematic drawing of mutant β subunits is shown in Figure 4A. CAT activities of the extract of BA/F3 cells transiently transfected with various hGMR mutants were determined (Figure 4B). As a positive control, CAT activity induced by mIL-3 via endogenous mIL-3R was used. CAT activity induced by hGM-CSF via wild-type hGMR is more than five times higher than that of the nonstimulated control and mutant 589 induced CAT activity to a level similar to that of the wild type. CAT activities of mutants 544 and 517 induced by hGM-CSF dropped to 30% and 20% of wild type, respectively, and CAT activity of mutant 455 was much the same as that of the unstimulated control. Box 1 and Box 2, consensus motifs of the cytokine receptor family, are located at amino acid (aa) positions 455–516 and 517–544, respectively. Taken together, these results suggest that Box 1 is essential for activation of the c-*myc* gene and that Box 2 has weak enhancing activity. A region at aa positions 544–589 that is located more distal to the transmembrane segment and is essential to activate the c-*fos* promoter, also showed strong enhancing activity for the c-*myc* promoter. Essentially the same results were obtained with the pmycXCAT reporter construct containing only the P2 minimal promoter.

Using BA/F3 cells stably expressing wild-type hGMR α subunit and various deletion mutants of β subunit, we previously had found that induction of endogenous c-*myc* mRNA is mediated via a region more proximal to the transmembrane region of the β subunit (Watanabe *et al.*, 1993b). The results described in the present report are consistent with those for the endogenous c-*myc* gene. However, it should be noted that the requirements of cytoplasmic regions of the hGMR β subunit for activation of c-*myc* promoter and for proliferation are not exactly the same. Previously, we reported that mutants 544 and 588 had comparable activities in stimulating cell proliferation (Watanabe *et al.*, 1993b).

Effects of Inhibitors of Tyrosine Kinase on Activation of the c-myc Promoter Induced by mIL-3 or hGM-CSF

We previously reported that the tyrosine kinase inhibitors herbimycin and genistein suppressed mIL-3– or hGM-CSF–induced proliferation and induction of c-*myc* mRNA, whereas induction of c-*fos* mRNA was rather resistant to these inhibitors (Watanabe *et al.*, 1993b). To confirm the involvement of tyrosine kinase in activation of the c-*myc* promoter in a transient system, the effects of herbimycin and genistein on CAT activity driven by the c-*myc* promoter were examined using BA/FGMR cells. Herbimycin at 0.5 μg/ml and genistein at 10 μg/ml suppressed mIL-3– or hGM-CSF–induced CAT activity of pmycCAT by 100% and 80%, respectively (Figure 5). Essentially the same results were obtained with pmycXCAT (data not shown). These results indicate that herbimycin- and genistein-sensitive tyrosine kinase(s) play an essential role in activation of the c-*myc* promoter induced by mIL-3 or hGM-CSF.



hGM-CSF	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+
mIL-3	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−
% conversion	6.0	13	39	8.0	16	46	6.2	16	14	6.9	18	9.4	7.0	17	7.1
hGM-CSFRβ	Wild			589			544			517			455		

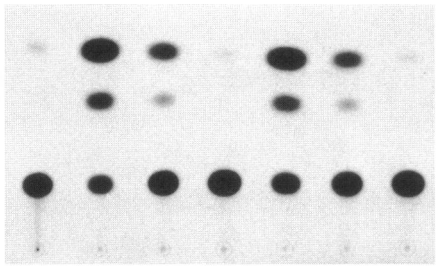
Figure 4. Requirement of the region of the hGMR β subunit to activate the c-*myc* promoter. (A) Schematic diagram showing various C terminus truncation mutants of hGMR β subunit. (B) Wild-type hGMR α subunit and various deletion mutants of β subunits were co-transfected with pmycCAT to BA/F3 cells. After depletion of mIL-3, either mIL-3 or hGM-CSF was added and CAT activities were analyzed as described in MATERIALS AND METHODS.

DISCUSSION

In this report, we used a transient transfection assay of c-*myc* promoter CAT plasmids and demonstrated that mIL-3 or hGM-CSF activates c-*myc* promoter in a hematopoietic cell line BA/F3. It has been difficult to monitor the response of the transfected c-*myc* gene to external stimuli whose promoter activity is relatively weak even at a fully induced level. We prepared several constructions of the c-*myc* promoter in different vectors using the CAT reporter gene, which gave a very low background. We also improved the protocol to achieve high efficiency of DNA transfection into BA/F3 or BA/FGMR cells and a transient assay of the c-*myc* promoter responding to mIL-3 or hGM-CSF was designed. To our knowledge, this is the first report of cytokine-dependent activation of the c-*myc* promoter in a transient system. This setup enabled delineation of the P2 promoter as the *cis*-regulatory element of the c-*myc* gene and signals required for activation of c-*myc* gene transcription by cytokines could be characterized. Definition of the P2 promoter as an mIL-3 or hGM-CSF responsive element is consistent with previous work showing the dominance of the P2 promoter in several types of cells (Spencer and Groudine, 1991).

Region of the β Subunit Required to Activate the c-myc Promoter

We previously showed that the signaling pathway of hGMR leading to proliferation and activation of the c-*myc* gene is distinct from that for activation of c-*fos* and c-*jun* genes, and the former and the latter depend on membrane proximal and distal regions of the β subunit, respectively. In the present work, we showed that the membrane proximal region (aa positions 455–544) is essential whereas the distal region (aa positions 544–589) has enhancing activity but is not essential for c-*myc* promoter activation. In our previous works, we focused on the membrane proximal region essential for cell proliferation and c-*myc* mRNA induction (Watanabe *et al.*, 1993b). Closer inspection of our previous Northern blot of c-*myc* mRNA revealed that the level of c-*myc* mRNA apparently decreased when the membrane distal region was removed even though BA/F544 retained the potential to induce c-*myc* mRNA. Hence, the involvement of two domains of the hGMR β subunit is similar to that obtained by the pmycCAT transient assay described in this paper. In our previous analysis, the region of the β subunit responsible for induction of c-*fos* and c-*jun* mRNAs was seen to be located between aa positions 626 and 763 (Watanabe *et al.*, 1993b). However, immunoprecipitation analysis using the anti-β subunit antibody revealed that the size of the β subunit of mutant 626 is much smaller than expected. We subsequently constructed mutant 589 and found that this mutant has the potential to activate c-*fos*–luciferase activity. It appears that the region of the β subunit responsible for induction of c-*fos* and c-*jun* mRNAs is located between aa positions 544 and 589 (T. Ito and S. Watanabe, unpublished result). This region is different from the one requiring more membrane proximal region between aa positions 455 and 544, which is essential for proliferation and activation of c-*myc* transcription. These results indicate that there are two distinct pathways in hGMR signaling.



Drugs	-	-	Genistein	Herbimycin	-	Genistein	Herbimycin
% conversion	5.6	79	22	5.3	70	21	5.0
stimulus	-	hGM-CSF			mIL-3		

Figure 5. Involvement of tyrosine kinases in mIL-3 or hGM-CSF induced c-*myc* transcription. pmycCAT activity induced by either mIL-3 or hGM-CSF was analyzed as described in the legend to Figure except that genistein (10 μg/ml) or herbimycin (0.5 μg/ml) was added 30 min or 24 h before the stimulation, respectively.

mIL-3 Regulates the c-myc Promoter through the E2F Complex

Interaction of E2F with other proteins such as DP-1 (La Thangue, 1994; Helin *et al.*, 1993), Rb (Nevins, 1992), and p107 (Cao *et al.*, 1992) has been shown to regulate transcription activity of E2F, in a positive or negative manner, depending on the nature of the interacting protein in the E2F complex. The E2F heterodimer complexed with p107 or Rb is suppressive to E2F-dependent transcription. For example, the p107/E2F complex interacts with thymidine kinase (TK) promoter and negatively affects its activation (Li *et al.*, 1993). Several E2F-related proteins consisting of families such as the human and mouse E2F1 and E2F2 and the human E2F3 have been reported (Li *et al.*, 1994). In vitro studies revealed that known E2F proteins interact with Rb but not with p107, thereby suggesting that an additional E2F protein(s) that is capable of associating with p107 may be present (Chittenden *et al.*, 1993; Dyson *et al.*, 1993). Isolation of the E2F4 gene from mice and humans whose product preferentially forms a complex with p107 and not with Rb is in keeping with this notion (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994). We performed gel supershift analysis using anti-E2F1 (KH95 or c-20) and anti-Rb (Ab-1 or Ab-2) antibodies and observed no shifted band. These results suggest that neither E2F1 nor Rb exist in the bands of BA/F3 extracts and further study is ongoing to identify the nature of the proteins.

We have no explanation as to why the p107/E2F complex decreases after mIL-3 stimulation. Phosphorylation of Rb has been shown to alter the potential to interact with other proteins. p107 is also a phosphoprotein but it is not clear whether or not phosphorylation of p107 alters its ability to interact with an E2F-like protein such as E2F4. Studies are ongoing to elucidate the regulation of phosphorylation of p107 by mIL-3. In contrast to the rapid decrease in the amount of p107/E2F heterodimer, kinetics of increase in free E2F is much slower. Addition of serum to quiescent cells activated synthesis of the E2F1 de novo (Slansky *et al.*, 1993) and the E2F site in the E2F1 promoter appears to play an important role in regulating the E2F1 gene (Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994). Whether or not growth factors or cytokines induce de novo synthesis of E2F4 protein remains unknown.

The Nature of Tyrosine Kinase(s) Responsible for Activation of the c-myc Promoter

The finding that herbimycin or genistein completely suppressed the c-*myc* promoter activity indicated the involvement of tyrosine kinase for activity. Recently, JAK family kinases have been shown to participate in cytokine receptor signaling and activation of JAK2 kinase by mIL-3 was reported (Silvennoinen *et al.*, 1993). Activation of JAK2 is associated with the membrane proximal region of the hGMR β subunit (Quelle *et al.*, 1994), but whether or not JAK kinase is involved in proliferation or c-*myc* mRNA induction after mIL-3 or hGM-CSF stimulation is unclear.

The transient transfection assay of the c-*myc* promoter described here, along with those of the c-*fos* promoter (Watanabe *et al.*, 1993a) and polyoma replication (Watanabe *et al.*, 1995) should prove to be useful in characterizing the signaling molecules required for c-*myc* promoter activation and proliferation.

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